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Title	High-pressure homogenisation combined with blanching to turn lettuce waste into a physically stable juice
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Abstract

Lettuce waste was blanched, ground, pre-homogenised at 40 MPa and subjected to high pressure homogenization (HPH) at 80 (1 pass) and 150 MPa (1, 10 passes) to obtain an ingredient intended for blended juice formulation. When lettuce was subjected to HPH without previous blanching, physically unstable juices were obtained. By contrast, the combination of HPH with a blanching pre-treatment allowed obtaining juices showing no physical separation and characterised by a bright green colour. This high stability was attributed to the modification of lettuce fibrous structure and to a 90% and 60% inactivation of polyphenoloxidase and pectin methylesterase, respectively. Juices presented a phenolic content of 3.5 ± 1.3 mg GAE/100 g and a microbial count at least 1 Log lower than that of corresponding not-blanched sample and below limits usually indicated for juice quality (4.7 Log CFU/g). During storage (4 °C), no phase separation was observed but microbial counts rapidly increased, suggesting the need for a further stabilization step.

Keywords	High pressure homogenization; lettuce; waste valorisation; juice; enzyme activity

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Dear Editor,

We send to your attention the research article entitled "**High-pressure homogenisation combined with blanching to turn lettuce waste into a physically stable juice**" by Stella Plazzotta and Lara Manzocco (Ref: IFSET_2018_1083), modified and corrected according to referees' suggestions.

Best regards,

Stella Plazzotta

Ref: IFSET_2018_1083
Title: High-pressure homogenisation combined with blanching to turn lettuce waste into a physically stable juice
Journal: Innovative Food Science and Emerging Technologies

Answer to the editors and reviewers:

Reviewer 1

1. In the abstract, the word "submitted to" may be changed to "treated with" or "subjected to". The word submitted is not appropriate to the context.

The word was changed, as suggested (line 7).

2. In introduction (line no. 28) "ready to eat..." may be changed to "ready to drink..."

The word was changed, as suggested (line 28).

3. The lines 37-39, 45-47 and 56-58 may be rephrased with the help of native English speaker/editor.

Sentences were rephrased, as suggested (lines 37-39, 49-51 and 62-64).

4. The sentence given in lines 62-69 may not be appropriate for introduction, those information may be included in results and discussion part in appropriate place.

Part of these lines was moved from the Introduction section to the Results and Discussion one (lines 202-206).

5. Lines 76- 84 are not needed in introduction. It may be included in Materials and Methods in appropriate form.

These lines were drastically reduced (lines 79-82), removing double information, already present in the Materials and Methods section.

6. Chapter 2.6 and 2.7 may be cut short, since the manuscript is not dealing any image processing related information

Chapters 2.6 and 2.7 were cut short, as suggested (lines 118-125).

7. In results and discussion, the chapter 3.1 and 3.2 may be splitted appropriately in to different paras in order to increase the readability or sub headings may be provided suitably.

Heading and text of section 3.1 were improved to increase clarity (lines 187-191). In addition, section 3.1 and 3.2 were split into different parts in order to increase the readability. Thus, proper sub headings were provided, as suggested.

Reviewer 2

1. Microbial load analysis immediately after HPH and over storage of 15 days is only provided for blanched samples. Is information on non-blanched lettuce juices for comparative reasons also available?

The storage test was only performed on blanched lettuce samples since not-blanched ones presented, immediately after HPH treatment (Table 3), a microbial load higher than that recommended for juice quality. Mention to this decision is reported in lines 345-348.

2. Energy input of HPH treatment should be stated.

As suggested by the reviewer, energy inputs were calculated and inserted in the paper. To this aim, energy density developed by HPH treatment was calculated as indicated in Materials and methods section (lines 107-113), reported in Table 1 and discussed in lines 191.

3. In section 3.1: Are you referring in this section only to the non-blanched samples? Please clarify.

Heading and text of section 3.1 were improved to increase clarity (lines 187-191). In addition, section 3.1 and 3.2 were split into different parts in order to increase the readability. Thus, proper sub headings were provided, as suggested by the referee 1.

4. Figure 2A: No phase separation for the non-blanched, grounded sample is displayed. Is this correct? Table 2 suggests a pronounced phase separation of the non-blanched, grounded sample. A picture of the blanched lettuce waste would be appreciated to stress that no phase separation at all is occurring.

As correctly remarked by the referee, no phase separation was observed in the Ground samples, independently on the application of blanching pre-treatment. The picture relevant to not-blanched ground sample clearly showed the presence of particle aggregates with different size. This was better described in lines 196-197. Nevertheless, upon storage for 24 h no visible phase separation was observed (line 257-258). As detailed in the text, this result could be attributed to the good ability of vegetable fibres to hold water (lines 258-260). Pictures of the two samples are shown in Table 2.

5. Figure 5: Why is data of control and HPH80 not shown? Please include the data to ease comparison.

As suggested by the referee, data relevant to HPH80 sample were added to Figure 5. Figure 5 focuses on lettuce juice microbial stability. For this reason, the reference sample was represented by the ground lettuce sample and not by lettuce leaves.

6. State microbial load quantitatively in abstract and the obtained reduction. Line 14 Please state the microbial limits for juice quality.

Requested information was added in the abstract (lines 14-15).

7. Line 29 freshlikelihood – use a more scientific term

The term was changed, as requested (line 30).

8. Line 41 quantify dietary fibres and polyphenol content

Requested information was added (line 41). In particular, data were retrieved from our previous publication: Plazzotta, S., Manzocco, L., & Nicoli, M. C. (2017). Fruit and vegetable waste management and the challenge of fresh-cut salad. *Trends in Food Science and Technology*, 63, 51–59.

9. Line 44 please formulate clearer

The sentence was rephrased (lines 46-51).

10. Line 45 what other techniques have been applied to improve the shelf life of freshly blended juices.

Requested information and relevant literature were added (lines 45-51).

11. Line 53 "…processing**,** such…"

The sentence was rephrased (line 56-60).

12. Lines 53 – 55 To clarify and support, it would be helpful to support this argument with literature.

Relevant literature was added (lines 62).

13. Line 113 Which color space was used? Hunter scale is named L, a, b. and CIELAB defined coordinates with L*, a* and b*- please specify.

The CIELAB scale was used. The mistake was corrected (line 117).

14. Line 126 Specify volume of cylinders and tested sample volumes.

Requested details were added (line 127).

15. Line 134 Please change the rpm quantification to rcf units to ease comparison with other literature.

The text was modified, as suggested (line 135).

16. Line 144 – 145 already includes results. Similarly in lines 156 – 157. Moreover, standard deviation of the PPO activity equals almost 90% of the measured value. Please provide reasoning for such a high standard deviation.

Lines reporting results relevant to PPO and PME activity were moved from Materials and Methods section to the Results and Discussion one (lines 239-240; 271-272). Typing error (0.004 and not 0.040) was corrected.

17. Line 156 – 157 Measuring pH difference per minute accurately with four digits after the comma seems unlikely.

A pH value with two conventional digits was measured and used to compute PME activity (lines 271-272). The latter was taken as the slope of the regression curve representing the pH as a function of time. PME activity, whose units are pH/min is presented as a value with four digits after the comma.

18. Line 160 which supernatant is referred to here?

The text was clarified, by adding the reference to the paragraph describing the supernatant preparation (line 159).

19. Line 163 UV-VIS instead of US-VIS

The text was modified (line 162).

20. Line 229 easily

The text was modified (line 244).

21. Line 331 – 333 Can you explain why the a* value for the 10x 150 MPa HPH treatment does not change at all upon storage and thus is even below the a* value of other samples after 15 days?

Discussion about the differences in greenness of ground samples and samples subjected to 1 pass HPH was improved (lines 220-223; 303-307). In addition, we hypothesised that the high temperature reached on multiple HPH passes promoted intense degradation of polyphenols and pigments during the treatment. As a consequence, negligible changes in juice colour were observed on further storage due to a low concentration of degradable pigments remaining in the sample. The text was clarified (lines 220-223, 303-307, 356-360, 364-365).

22. Line 349 What is an interesting phenol content – please specify more scientifically.

The term “interesting” was changed in “partially maintained” (line 381).

23. Line 356 – 357 Please support your comments on increasing usage of HPH as processing operation in industry, good feasibility and cost effectiveness of HPH with references.

In order to support these conclusive comments without adding references in the Conclusion section, the Introduction section was integrated with literature data relevant to the increasing industrial usage of HPH (lines 56-60).

24. How can 1.7 Log CFU/g be the detection limit but values for Log CFU/g are reported as 1.70 ± 0.22 Log CFU/g?

Microbial enumerations and statistical elaboration were checked. Table 3 was corrected accordingly.

HPH of blanched lettuce waste produces physically stable bright-green juices

HPH of blanched lettuce waste leads to partial retention of polyphenols

Blanching and HPH do not allow lettuce waste juice microbial stabilization

Industrial application: Solid waste generated by fresh-cut processing of lettuce could be valorised by the application of blanching and HPH, leading to an innovative ingredient potentially exploitable in the formulation of healthy blended juices, smoothies and comminuted food. This effort is worth making considering that HPH is being increasingly introduced as processing operation in various industrial contexts, showing good feasibility and cost effectiveness, and could allow valorisation of different leaf discards.

High-pressure homogenisation combined with blanching to turn lettuce waste into a physically stable juice

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Abstract

Lettuce waste was blanched, ground, pre-homogenised at 40 MPa and subjected to high pressure homogenization (HPH) at 80 (1 pass) and 150 MPa (1, 10 passes) to obtain an ingredient intended for blended juice formulation. When lettuce was subjected to HPH without previous blanching, physically unstable juices were obtained. By contrast, the combination of HPH with a blanching pre-treatment allowed obtaining juices showing no physical separation and characterised by a bright green colour. This high stability was attributed to the modification of lettuce fibrous structure and to a 90% and 60% inactivation of polyphenoloxidase and pectin methylesterase, respectively. Juices presented a phenolic content of 3.5 ± 1.3 mg GAE/100 g and a microbial count at least 1 Log lower than that of corresponding not-blanched sample and below limits usually indicated for juice quality (4.7 Log CFU/g). During storage (4 °C), no phase separation was observed but microbial counts rapidly increased, suggesting the need for a further stabilization step.

Industrial application: Solid waste generated by fresh-cut processing of lettuce could be valorised by the application of blanching and HPH, leading to an innovative ingredient potentially exploitable in the formulation of healthy blended juices, smoothies and comminuted food. This effort is worth making considering that HPH is being increasingly introduced as processing operation in various industrial contexts, showing good feasibility and cost effectiveness, and could allow valorisation of different leaf discards.

Keywords:
High pressure homogenization, lettuce, waste valorisation, juice, enzyme activity

1 Introduction

Increasing consumer demand for low-caloric foods with fresh-like characteristics and high nutritional quality has encouraged the research of alternative vegetable products. In this context, ready to drink juices, smoothies and enriched beverages are experiencing an increasing market demand due to their fresh-like sensory attributes, health benefits, convenience and clean-label (Yi et al., 2018). Among these products, blends of freshly extracted fruit and vegetable juices offer the possibility to develop new products, which present innovative flavours and improved nutritional quality, due to the high concentration of fibres and antioxidants as well as to the low caloric content (De Carvalho, Maia, De Figueiredo, De Brito, & Rordrigues, 2007). Vegetables used to produce commercial blended juices include tomato and carrot but also leaf-vegetables such as spinach, celery, kale and parsley (Hao, Zhou, Koutchma, Wu, & Warriner, 2016).

Fresh-cut processing of *Iceberg* lettuce generates huge waste amounts (up to 50% of the initial lettuce head weight). Lettuce waste is currently transported to centralized plants, where it is co-composted or co-digested with other organic wastes. Beside requiring high transport and disposal costs, these management strategies are not able to properly valorise lettuce waste. The latter, in fact, presents a high content in dietary fibres (28.9 g/100 g dry weight) and polyphenols (1.9 mg GAE/g dry weight), and can be supplied continuously and in large quantity by the fresh-cut industry (Plazzotta, Manzocco, & Nicoli, 2017). For these reasons, lettuce waste could be considered an interesting raw material for producing healthy blended juices.

Different formulation and processing strategies have been investigated in order to guarantee an adequate shelf-life of fresh blended juices, which is limited by microbial, enzymatic and physical alterations. Formulation strategies include water activity reduction, nutrient restriction, acidification and use of anti-

48 microbial additives (Raybaudi-Massilia, Mosqueda-Melgar, Soliva-Fortuny, & Martín- Belloso, 2009).
49 Among process innovations, high hydrostatic pressure is an established technique for fresh juices
50 processing, since it allows microbial and enzymatic stabilization, while maintaining their nutritional and
51 sensory characteristics (Deliza, Rosenthal, Abadio, Silva, & Castillo, 2005). However, this technology
52 cannot provide an adequate physical stability of the juices, which undergo rapid phase separation during
53 storage (Laboissière et al., 2007). Although the addition of hydrocolloids (e.g. pectin, carboxymethyl
54 cellulose, and sodium alginate) have been repeatedly suggested to control juice sedimentation (Ibrahim
55 et al., 2011), this strategy hardly fits with consumer expectations for clean label products, leading to the
56 need for alternative solutions. In this regard, high-pressure homogenization (HPH) is a promising
57 technique, particularly suitable for continuous production of fluid foods and increasingly introduced as
58 processing operation in different industrial contexts, showing a good scaling-up potential (Calligaris,
59 Foschia, Bartolomeoli, Maifreni, & Manzocco, 2012; Martínez-Monteagudo, Yan, & Balasubramaniam,
60 2017). In particular, HPH has been demonstrated to represent a valid alternative to cloudiness
61 preservatives, due to its ability of modifying the structure-forming properties of plant fibre suspensions
62 in the juice (Bengtsson & Tornberg, 2011; Van Buggenhout et al., 2015). HPH treatments, in fact, not
63 only reduce the size of suspended particles, decreasing sedimentation rate, but also modify fibre physico-
64 chemical properties, such as water holding, swelling and structuring capacity (Van Buggenhout et al.,
65 2015). HPH has been shown to be particularly effective in physical stabilization of different products,
66 including tomato and banana juices (Calligaris et al., 2012; Colle, Van Buggenhout, Van Loey, &
67 Hendrickx, 2010). Pressures between 50 and 150 MPa are generally applied for juice HPH treatment and
68 the juice can also be recirculated in the homogenizer to increase treatment intensity without necessarily
69 increase treatment pressure (Karacam, Sahin, & Oztop, 2015; Yi et al., 2018).
70 Although the recognised efficacy of HPH in reducing particle size of vegetable suspensions, tissue
71 disruption is often responsible for a rapid juice colour depletion, due to the activity of oxidative enzymes

on phenols and natural-occurring pigments (Liu, Liu, Liu, et al., 2009). The inactivation of these enzymes by the application of blanching prior to HPH can allow obtaining a colour-stable product. In this case, the vegetable is subjected to a "heat-shock" treatment, during which a heat treatment is rapidly followed by a quick cooling of the product. Plant tissue enzymes are thus inactivated, leading to a reduced colour change upon further HPH treatment and storage (Devece et al., 1999).

The objective of the current work was to evaluate if HPH, combined with blanching, could be used to turn lettuce waste into a value-added ingredient, possibly exploitable in the formulation of blended juices, smoothies and comminuted food. Hereto, the work was divided in three parts. In the first one, lettuce waste was subjected to different homogenisation treatments. In the second part, the effect of a blanching pre-treatment before homogenisation was evaluated. Finally, a storage test was performed on blanched lettuce juices.

2 Materials and methods

2.1 Lettuce waste preparation

A 10-kg batch of *Iceberg* lettuce (*Lactuca sativa* var. *capitata*) was purchased at the local market and stored overnight at 4 °C. After removal of bruised and spoiled parts, outer leaves were manually removed from lettuce heads, simulating operations that are industrially carried out during fresh-cut lettuce processing. Lettuce waste amounted to 274 ± 23 g/kg of the entire processed lettuce, which is in agreement with amounts commonly collected in a fresh-cut lettuce head process. Lettuce waste was washed with flowing water (18 ± 1 °C) and sanitized 20 min in a chlorinated bath containing 200 mg/L of NaClO with a 100 g/L lettuce/water ratio. Lettuce waste was then rinsed with flowing water and centrifuged in a manual kitchen centrifuge (mod. ACX01, Moulinex, France) for 1 min (Plazzotta et al., 2017).

2.2 Blanching

95 Lettuce waste was immersed in water (100 g/L lettuce/water ratio) at 90 °C for 30 s and then immediately
96 placed 1 min into an ice bath (100 g/L lettuce/water ratio). After that, lettuce waste was accurately dried
97 using absorbing paper and stored at 20 °C for 10 min before treatment.

98 *2.3 Grinding*

99 Lettuce waste was ground using a domestic grinder (MC3001, Moulinex, Milan, Italy) at ambient
100 temperature for 5 min.

101 *2.4 High pressure homogenisation (HPH)*

102 A continuous lab-scale high-pressure homogeniser (Panda Plus 2000, GEA Niro Soavi, Parma, Italy)
103 supplied with two PS type homogenisation valves with a flow rate of 10 L/h was used. Ground lettuce
104 waste (150 g) was pre-homogenised at 40 MPa to reduce valve obstruction risk. High pressure
105 homogenisation treatments were then conducted at 80 and 150 MPa. Moreover, at 150 MPa, 10
106 subsequent cycles were performed. The different combinations of treatments performed on lettuce waste
107 and the identification of sample names are reported in Table 1. The energy density (E_V, J/g) transferred
108 from the homogenisation valve to the sample was determined as described by Stang, Schuchmann, and
109 Schubert (2001), according to eq. 1:

110 $E_V = \Delta P$ (eq. 1)

111 where ΔP is the pressure difference operating at the valve (MPa). The energy density of multiple passes
112 HPH was calculated as the sum of the energy density values of the corresponding single HPH pass
113 (Calligaris et al., 2016). The energy density developed by HPH treatments is reported in Table 1.

114 *2.5 Colour*

115 Colour was determined using a tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka,
116 Japan) equipped with a CR-300 measuring head. The instrument was standardized against a white tile.
117 Colour was expressed in L*, a* and b* CIELAB scale parameters.

2.6 Image acquisition

Images were acquired using an image acquisition cabinet (Immagini & Computer, Bareggio, Italy) equipped with a digital camera (EOS 550D, Canon, Milan, Italy) and 4 frosted photographic floodlights (23 W).

2.7 Optical microscopy

Samples were observed at room temperature using a Leica DM 2000 optical microscope, images taken at 200X magnification using a Leica EC3 digital camera and elaborated with the Leica Suite Las EZ software (Leica Microsystems, Heerburg, Switzerland).

2.8 Phase separation

Samples (40 mL) were poured in 50 mL-graduated cylinders for 24 h at 4 °C. Phase separation was visually assessed and expressed as volume percentage of separated phase.

2.9 Viscosity

Rheological analyses were performed using a RS6000 Rheometer (Thermo Scientific RheoStress, Haake, Germany), equipped with a Peltier system for temperature control. Measures were performed using a bob-cup geometry at 20 °C. Flow curves were recorded increasing shear rate from 0.1 to 100 s^{-1}.

2.10 Supernatant preparation

Samples were poured in 1.5 mL Eppendorf tubes and the supernatant was collected after centrifugation (Hittich MIKRO 20, Centrifuge, Tuttlingen, Germany) at 8518 g for 15 min.

2.11 Polyphenoloxidase activity

The polyphenoloxidase (PPO) activity was assayed spectrophotometrically (Shimadzu UV-2501PC, UV-Vis recording spectrophotometer, Shimadzu Corporation, Kyoto, Japan) at 25 °C according to the methodology of Kahn (1985). The reaction was started by the addition of 200 µL of supernatant to 1.8

mL of 0.1 mol/L potassium phosphate buffer pH 7 and 1.5×10^{-3} mol/L L-Dopa (Carlo Erba, Milan, Italy). The absorbance at 420 nm was monitored every 10 s for 10 min. The changes in absorbance per min were calculated by linear regression, applying the pseudo zero order kinetic model. The eventual final stationary phase was excluded from regression data. The slope of the very first linear part of the reaction curve was used to determine PPO activity (k_{PPO}). PPO activity was expressed as the percentage activity as compared to that of the Ground sample not subjected to blanching or HPH treatments (Table 1).

2.12 Pectin methylesterase activity

Pectin methylesterase (PME) activity was measured using the method described by Martin-Diana et al. (2005) with some modifications. Briefly, the initial pH of 10 g of sample (3.1 ± 0.3) was adjusted at 7.5 using NaOH 1 M (Carlo Erba, Milan, Italy). After that, 0.2 mL of NaOH 0.05 M were added, and during the time required by each sample to reach again a pH value of 7.5, pH was continuously monitored using a pHmeter (pH-Meter BASIC 20, Crison, Barcelona, Spain) equipped with a measuring head for liquids (52 02, Crison, Barcelona, Spain). The changes in pH per min were calculated by linear regression, applying the pseudo zero order kinetic model. The eventual final stationary phase was excluded from regression data. The slope of the very first linear part of the reaction curve was used to determine PME activity (k_{PME}). PME activity was expressed as the percentage activity as compared to that of the Ground sample not subjected to blanching or HPH treatments (Table 1).

2.13 Total polyphenolic content

Total polyphenolic content (TPC) was determined using Folin-Ciocalteau reagent (Singleton & Rossi, 1965). The reaction mixture contained 50 µL of supernatant (paragraph 2.10), 2 mL distilled water and 250 µL of the Folin-Ciocalteau reagent. After 1 min, 1 mL of a sodium carbonate-water solution (0.15 g/mL) was added and the solution was mixed using a vortex for 30 s (MIX10, Falc Instruments, Treviglio, Italy). After 2 h reaction at ambient temperature, mixture absorbance was read at 750 nm using UV-Vis

spectrophotometer (Shimadzu UV-2501PC, UV-Vis recording spectrophotometer, Shimadzu Corporation, Kyoto, Japan). A calibration curve was made with standard solutions of gallic acid in the range 0.1-1000 mg/L ($R^2 = 0.99$). Results were expressed as mg of gallic acid equivalents (GAE) per 100 g of sample.

2.14 Microbial analyses

For microbiological analyses, 25 g of Control sample was diluted with 100 mL Maximum Recovery Diluent (Oxoid, Basingstoke, UK) and homogenised for 1 min in a Stomacher (PBI International, Milan, Italy). By contrast, Ground, Pre-homogenized, HPH 80, HPH 150 and HPH 150x10 samples (Table 1) were directly used. Serial dilutions of each suspension were made in Maximum Recovery Diluent (Oxoid) and analysed for microbial counts. Appropriate aliquots (0.1 or 1 g) were spread on agar plates. Plate Count Agar (Oxoid) and Man Ragosa Sharpe (MRS) were used for enumeration of total bacterial count and lactic acid bacteria respectively, and plates were incubated for 48 h at 30 °C. Oxytracycline-Glucose- Yeast Extract (OGY) agar (Oxoid), was used for enumeration of yeasts, and plates were incubated for 72 h at 28 °C.

2.15 Sample storage

Aliquots of 50 mL of sample were introduced in sterile falcon tubes and stored for up to 15 days at 4 °C in a refrigerated cell. At increasing time during storage, samples were removed from the refrigerator, equilibrated at 22 °C and analysed.

2.16 Data analysis

Analyses were carried out at least three times in two replicated experiments. Analysis of variance ($p<0.05$) and linear regression analysis were performed using R (The R foundation for statistical computing, v.3.1.1).

3 Results and discussion

3.1 Effect of high pressure homogenisation without blanching pre-treatment

In the first part of the study, the effect of HPH treatments on lettuce waste was investigated. Hereto, lettuce waste was subjected to grinding, equilibrated at room temperature, and pre-homogenised at 40 MPa, to avoid valve blockage. The obtained lettuce dispersion was then homogenised at 80 and 150 MPa, the latter treatment being applied for 1 or 10 cycles. Based on eq. 1, samples were thus subjected to increasing energy densities from 40 up to 1540 J/g (Table 1). Grinding and pre-homogenisation increased sample temperature by 2 and 5 °C, respectively. By contrast, further HPH application resulted in a progressive temperature increase, so that samples reached 38, 65 and 85 °C after treatments at 80, 150 and 150 MPa applied for 10 passes, respectively.

3.1.1 Tissue structure

Ground lettuce waste showed a non-homogenous appearance due to the presence of particle aggregates with different size. The visual homogeneity of samples progressively increased with the HPH treatment intensity, as also confirmed by microscopic images (Table 2). Tissue cellular organization was well-evident in both Control and Ground samples. The 40 MPa-pre-homogenised sample still presented a number of intact cells, although the broken cell material was the most abundant. No intact cells were observed in samples subjected to HPH treatments (HPH 80, HPH 150, HPH 150x10), in which the broken cell material appeared uniformly distributed. HPH is well-known to promote vegetable tissue disruption, due to the highly energetic phenomena taking place during the treatment. During HPH, in fact, the fluid is forced to pass through a narrow gap, leading to rapid acceleration followed by sudden pressure drop. In this way, the fluid undergoes simultaneous intense stresses, including elongational forces, cavitation and turbulent flow (Stang et al., 2001). In this regard, similar disruptive effects have been reported upon HPH treatment of tomato and algae (Bot et al., 2017; Samarasinghe, Fernando, Lacey, & Faulkner, 2012).

3.1.2 Colour

Grinding promoted a visible change in lettuce colour, as confirmed by the sensible decrease in luminosity (L*) and yellow point (b*), and the concomitant increase of red point (a*) (Table 2). These results suggest a significant loss of the original lettuce green colour, in favour of a brownish one. The application of 40 MPa pre-homogenisation and HPH treatments up to 150 MPa, inverted this tendency, leading to a bright green colour, as suggested by the increase in L* and the decrease of a*. By contrast, upon 10 passes at 150 MPa, samples tended again to become more brownish (Table 2). Such results can be attributed to the effect of different phenomena taking place simultaneously. The significant browning induced by grinding can be attributed to the decompartmentalization of oxidative enzymes and their phenolic substrates upon tissue disruption, leading to polymerized brown derivatives (Espín, Jolivet, & Wichers, 1998). Tissue disruption was further promoted by pre-homogenization and HPH, as well-evidenced by microscopic images (Table 2), resulting in smaller particles with higher surface area, which promoted an increase in light scattering and thus in sample luminosity (L*) (Ahmed, Shivhare, & Raghavan, 2000). In addition, HPH has been reported to effectively release chlorophyll from intracellular spaces (Carullo et al., 2018), possibly accounting for the greener colour (lower a* value) of samples subjected to one HPH pass up to 150 MPa as compared to Ground sample. The further loss of green upon 10 passes at 150 MPa can be possibly attributed to the pronounced thermal effect of this treatment, leading to the degradation of both chlorophyll and polyphenols (Espín et al., 1998; Koca, Karadeniz, & Burdurlu, 2007).

3.1.3 Total phenolic content and polyphenoloxidase activity

To further study the role of oxidative phenomena upon HPH treatment of lettuce waste, total phenolic content (TPC) and polyphneoloxidase (PPO) activity were evaluated (Figure 1). Despite the inherent vegetable variability and the application of different extraction parameters and quantification methods, the TPC value of the Ground sample (about 14 mg GAE/100 g of lettuce waste) (Figure 1) resulted in

232 the range reported in the literature for green-leaf lettuce. In this regard, a TPC of 18 and 14 mg/100 g
233 fresh weight were obtained by Llorach, Martínez-Sánchez, Tomás-Barberán, Gil, & Ferreres (2008) and
234 Llorach, Tomás-Barberán, & Ferreres (2004), respectively. A TPC value similar to that observed in the
235 Ground sample was also found upon the 40 MPa-pre-homogenisation treatment of lettuce waste. By
236 contrast, a further increase in HPH intensity up to 150 MPa led to a progressive reduction in the TPC,
237 while the application of 10 passes at 150 MPa did not promote a further phenol loss (Figure 1A). This
238 can be attributed to both the thermal effect of HPH treatment, leading to phenol degradation and the
239 activation of PPO (Figure 1B). In particular, Ground sample presented a PPO activity of 0.045 ± 0.004
240 ΔAbs/min and a 60, 90 and 40% enzymatic activity increase was observed in Pre-homogenised, HPH 80
241 and HPH 150 samples, respectively. The HPH-induced PPO activation can be due to multiple effects of
242 the treatment. Firstly, it can be inferred that, upon cell disruption induced by HPH (Table 2), PPO, which
243 has been reported to be highly active in *Iceberg* lettuce, was no longer separated from its phenolic
244 substrates, which were thus easily oxidised (Mai & Glomb, 2013). In addition, lettuce cell disruption has
245 been reported to promote the release of proteases, responsible for the activation of latent PPO which,
246 differently from the free soluble one, is bounded to the cellular membrane (Cantos, Espín, & Tomás-
247 Barberán, 2001). In addition, HPH processing is well-known to affect PPO conformation and activity. In
248 this regard, a progressive PPO activation was also observed in Chinese pear and mushroom subjected to
249 high pressure microfluidisation at pressures in the range from 80 to 200 MPa (Liu, Liu, Liu, et al., 2009;
250 Liu, Liu, Xie, et al., 2009). Figure 1B also shows that only the application of 10 passes at 150 MPa led
251 to an almost complete PPO inactivation, possibly explaining the lack in further TPC reduction (Figure
252 1A). However, in this case, the intense PPO inactivation should be mainly attributed to the fact that
253 multiple HPH passes made temperature sample exceed that of PPO inactivation (70 °C) by about 15 °C
254 (Terefe, Delon, Buckow, & Versteeg, 2015).

3.1.4 Physical stability and pectin methylesterase activity

To evaluate the physical stability of samples, phase separation after 24 h of refrigerated storage was assessed (Figure 2A). Interestingly, the Ground sample showed no phase separation, while the latter increased with the applied pressure. These results can be possibly due to the effect of HPH on network-forming ability of vegetable fibres. It is likely that in Ground sample, lettuce fibres tended to intertwine and form a network trapping the free liquid which would otherwise separate from the product. By disrupting the vegetable components (Table 2), HPH reduced the length of lettuce fibres to a point where they were no longer able to form an effective network. In this regard, Colle et al. (2010) highlighted a significant change in water holding capacity of tomato fibres subjected to HPH up to 130 MPa. Although the sample obtained by the 150 MPa treatment for 10 passes showed the highest tissue disruption (Table 2), it presented a lower phase separation as compared to the 150 MPa single-pass treatment. In addition to the effect of fibre length, an effect of applied treatments on pectolytic enzymes can be accounted for the observed separation data. In fact, as a consequence of the activity of these enzymes, cross-linking between carboxyl groups in pectin molecules are favoured, leading to structure changes in vegetable derivatives. In particular, the activity of pectin methylesterase (PME) has been reported to destroy the cloudy stability in citrus fruit juices (Welti-Chanes, Ochoa-Velasco, & Guerrero-Beltrán, 2009) and to favour crispness loss in *Iceberg* lettuce (Martin-Diana et al., 2005). The Ground sample presented a PME activity of -0.0472 ± 0.0002 ΔpH/min and HPH treatments up to 150 MPa reduced enzymatic activity by about 20% (Figure 2B). The application of 10 passes at 150 MPa led to a further inactivation, leading to a residual PME activity around 50%. It is thus likely that only the most intense treatment promoted a sufficient PME inactivation to reduce separation phenomena. This agrees with results obtained by Welti-Chanes et al. (2009) in orange juice, in which the application of 5 passes at pressures up to 250 MPa was shown to enhance PME inactivation.

3.1.5 *Microbial load*

Finally, samples were analysed for total bacterial (TBC), yeast and lactic acid bacteria (LAB) counts (Table 3). LAB resulted always lower than detection limit (1.7 CFU/g), while TBC and yeasts progressively decreased with HPH intensity. Only the 150 MPa treatments were able to attain lettuce juices presenting microbial loads below limits usually indicated for vegetable and fruit juice quality (3.7-4.7 Log CFU/g for TBC) (Simforian, Nonga, & Ndabikunze, 2015). Such result should be attributed not only to HPH effect, but also to the intense heating promoted by the treatment (Comuzzo et al., 2017).

3.2 *Effect of high pressure homogenisation with blanching pre-treatment*

The application of HPH treatments to lettuce waste resulted in juices showing a critical instability of physical and microbiological parameters. In the light of these findings, in the second part of the study, the possibility to improve the stability of HPH treated lettuce waste by the application of a blanching pre-treatment was evaluated.

3.2.1 *Tissue structure*

The visual appearance and the microscopic structure of obtained samples is reported in Table 2. As compared to not-blanched samples, Ground blanched ones presented an apparently more homogeneous structure. Microscopic images revealed that a good cellular disruption was obtained also by the application of grinding and that HPH treatments further increased the homogeneity of the sample, which showed uniformly distributed cellular content (Table 2). Blanching treatment actually promotes cell turgidity loss and cell wall degradation, leading to a more deformable and softer texture, which is expected to favour grinding and homogenisation (Xu, Yu, & Li, 2015).

3.2.2 *Colour*

As compared to not-blanched samples, blanched ones presented similar L* and b* values but significantly lower a* data. As expected, blanching hindered browning phenomena, allowing to better

maintain the original lettuce green colour. This can be attributed to the inactivation effect of blanching on oxidative enzymes, responsible for polyphenol oxidation and chlorophyll degradation (Devece et al., 1999). Similar to not-blanched samples (Table 2), also the blanched ones obtained by the application of single-pass HPH treatments presented a greener colour (lower a* value) than the blanched Ground sample, due to HPH-induced chlorophyll extraction (Carullo et al., 2018). Reversely, as already pointed out (paragraph 3.1.2), the application of 10 passes at 150 MPa led to colour bleaching, due to the intense thermal effect of this treatment.

3.2.3 Total phenolic content and polyphenoloxidase activity

To this regard, Figure 1B shows that blanched samples presented PPO activity always lower than 14%. The only blanched sample presenting a higher a* value (and thus a lower green point) was the one obtained by 10 passes at 150 MPa. This can be explained by the intense heating upon such treatment, possibly leading to severe thermal degradation of both polyphenols and chlorophyll (Manzocco, Mastrocola, Nicoli, & Marangoni, 2001; Weemaes, Ooms, Van Loey, & Hendrickx, 1999). Beside colour, PPO inactivation also affected the phenolic content of HPH treated lettuce waste. As shown in Figure 1A, TPC of blanched samples as a function of HPH treatment intensity followed an opposite pattern as compared to not-blanched ones, resulting in progressively higher TPC values. However, given the treatment, TPC of blanched samples resulted always lower than that of not-blanched ones. This apparently contrasting result can be explained considering the counterbalancing effect of blanching and HPH on phenol content. Vegetable blanching is known to cause significant depletion in phenolic content, due to both applied temperature and leaching effect in the water used for the treatment (Eyarkai Nambi, Gupta, Kumar, & Sharma, 2016). However, HPH-induced tissue disruption (Table 2) promoted the extraction from blanched lettuce cells of not leached phenolic compounds that, in the absence of an intense oxidative PPO activity, were largely maintained. In this regard, HPH has been extensively used

as cell-breakage technology favouring extraction of different target molecules from vegetable tissues (Zhu et al., 2016).

3.2.4 Physical stability, viscosity and pectin methylesterase activity

Blanched samples resulted physically stable after 24 h refrigerated storage, showing no visible phase separation (Figure 2A). Such result can be partially attributed to the effect of blanching on pectolytic enzymes. As shown in Figure 2B, in fact, blanched samples presented a PME activity always lower than 40% and progressively decreasing with the increase of HPH intensity. Blanching at temperatures higher than 80 °C has been actually reported to inactivate PME in different vegetables (Ni, Lin, & Barrett, 2005). Nevertheless, microscopic images evidenced a considerable HPH effect on blanched lettuce structure (Table 2), that was evaluated by means of rheological measurements (Figure 3). Sample viscosity decreased with the increase in HPH treatment intensity. Similar results were also reported for apple and banana juices and can be possibly attributed to the HPH-induced reduction of fibre dimension, favouring fibre-fibre interaction rather than fibre-water ones (Colle et al., 2010).

3.2.5 Microbial load

Finally, the microbial quality of blanched samples was determined (Table 3). LAB and yeasts resulted always lower than detection limit. The TBC of Ground sample resulted lower than 3.5 Log CFU/g and progressively decreased with the HPH intensity. These values resulted not only lower than those of not-blanched samples, but also below limits usually indicated for vegetable and fruit juice quality (3.7-4.7 Log CFU/g for TBC). Blanching, in fact, can reduce the microbial load of vegetable surface, due to the applied temperature and microorganism leaching into treatment water (Xiao et al., 2017).

3.2.6 Storage test

Based on the obtained results, HPH treatments associated to a blanching pre-treatment would allow obtaining a lettuce juice presenting good physical stability and acceptable microbial load, potentially

exploitable for the formulation of blended juices, smoothies and comminuted foods. These samples were thus selected for a storage test that was conducted in refrigerated conditions up to 15 days. During this period, no significant changes in L* and b* parameters of the samples were observed (data not shown). However, in the Ground sample and in the samples subjected to a single HPH pass a progressive increase in a* was observed, indicating sample browning (Figure 4). This effect should be probably attributed the progressive degradation of chlorophyll during storage (Perucka, Olszówka, & Chilczuk, 2014) and the formation of oxidised polyphenols upon their chemical interaction with oxygen (Le Bourvellec, Le Quéré, Sanoner, Drilleau, & Guyot, 2004), rather than to PPO activity. The latter was actually very low in the just prepared samples (Figure 1B) and remained below 8% (data not shown) during the entire storage test, indicating that applied treatments were able to irreversibly inactivate this enzyme. At the end of storage, the a* value of samples subjected to one HPH pass resulted lower than that of Ground sample. This higher greenness was already observed immediately after the treatment (Table 2) and possibly attributed to the ability of HPH to release intracellular compounds, such as chlorophyll (paragraph 3.2.2). Only the sample obtained by 10 passes at 150 MPa showed no changes in a* during storage. As already pointed out, this sample presented, immediately after the treatment, an a* value significantly higher than that of other blanched samples (Table 2). Thus, it is likely that the high temperature reached on multiple HPH passes promoted intense degradation of polyphenols and pigments during the treatment. This, in turn, led to negligible changes in juice colour on further storage, probably due to a low concentration of degradable pigments remaining in the sample. No phase separation neither viscosity changes were observed during the 15 day-storage (data not shown) in agreement with PME irreversible inactivation by applied treatments. In fact, PME showed no changes in activity during storage time (data now shown).

Figure 5 shows the evolution of microbial load during time. Yeasts resulted always lower than detection limit (1.7 UFC/g). Except for HPH 150x10 sample, in which LAB growth resulted inhibited, TBC and

LAB progressively increased in all samples (Figure 5A and B), exceeding values commonly indicated for fruit and vegetable juice quality (3.7-4.7 Log CFU/g for TBC) after only 3 days. As widely reported in the literature, HPH presents a reduced antimicrobial efficacy and is thus usually combined with other treatments (e.g. acidification, high hydrostatic pressure) to attain a microbiologically stable product (Georget, Miller, Callanan, Heinz, & Mathys, 2014; Patrignani, Tabanelli, Siroli, Gardini, & Lanciotti, 2013).

4 Conclusions

Results obtained in this study suggest that high pressure homogenisation might be an interesting technology to fully exploit lettuce waste and obtain innovative healthy ingredients to be further used in food production. The combination of this technology with a blanching pre-treatment resulted in a homogeneous lettuce juice, presenting a partially maintained phenol content, a bright green colour, and high physical stability during storage. Although promoting an irreversible inactivation of alterative enzymes, the proposed treatment was not adequate for guaranteeing juice microbial stability. HPH of blanched lettuce should be thus associated to a further stabilization step (e.g. acidification or high hydrostatic pressure), reasonably applied at the level of the final formulation, being a blended juice, a smoothie or a comminuted food. Although the case here presented was relevant to lettuce waste, obtained results could be easily extended to other leaf vegetables and discards, largely broaden their applicability and impact. This effort is worth making considering that HPH is being increasingly introduced as processing operation in different industrial contexts, showing good feasibility and cost effectiveness.

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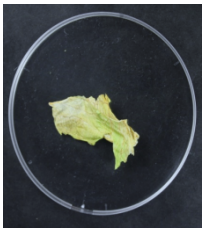
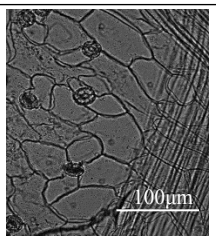
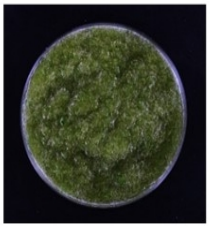
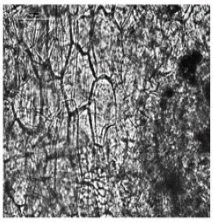
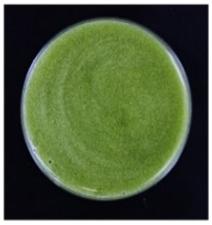
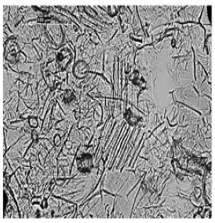
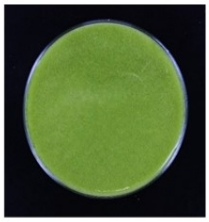
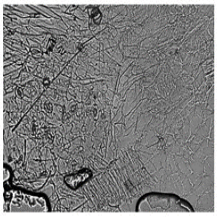
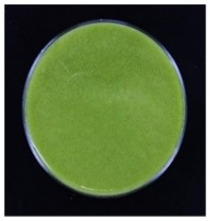
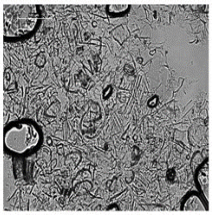
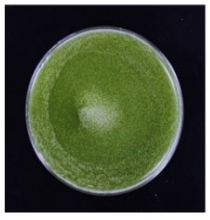
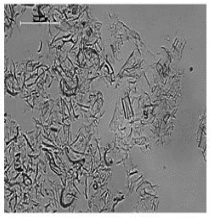
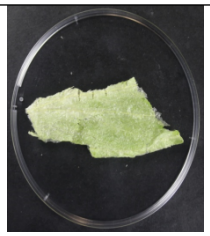
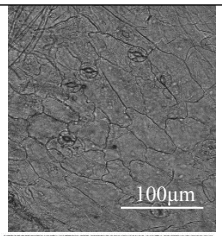
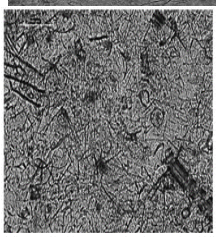
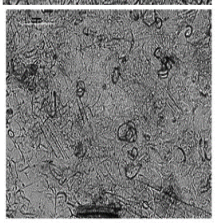
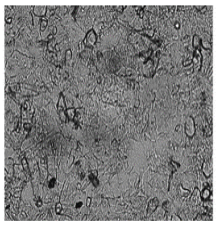
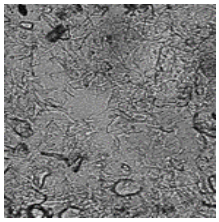
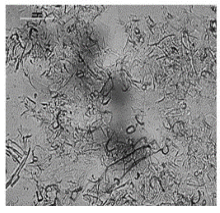
Table 1. Pre-treatments and treatments performed on lettuce waste and obtained samples. The enrgy density (E_V) developed by high pressure homogenisation (HPH) tretaments is also reported.

Sample		Pre-treatment				Treatment		E_V (J/g)
		Blanching	Grinding	Pressure (MPa)	Passes	Pressure (MPa)	Passes	
Not-blanched	Control	no	no	/	/	/	/	/
	Ground	no	yes	/	/	/	/	/
	Pre-homogenised	no	yes	40	1	/	1	40
	HPH 80	no	yes	40	1	80	1	120
	HPH 150	no	yes	40	1	150	1	190
	HPH 150x10	no	yes	40	1	150	10	1540
Blanched	Control	yes	no	/	/	/	/	/
	Ground	yes	yes	/	/	/	/	/
	Pre-homogenised	yes	yes	40	1	/	1	40
	HPH 80	yes	yes	40	1	80	1	120
	HPH 150	yes	yes	40	1	150	1	190
	HPH 150x10	yes	yes	40	1	150	10	1540

Table 2. Visual appearance, microscopic image, and colour of not-blanched and blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes.

Sample		Appearance	Microscopy	Colour		
				L*	a*	b*
Not-blanched	Control			71.4 ± 1.3[a]	-16.9 ± 1.2[a]	31.6 ± 1.4[a]
	Ground			39.8 ± 0.7[f]	-3.5 ± 0.3[e]	17.0 ± 1.0[d]
	Pre-homogenised			43.6 ± 0.3[e]	-4.3 ± 0.1[c]	20.7 ± 1.0[c]
	HPH 80			48.6 ± 0.1[c]	-6.2 ± 0.1[b]	26.1 ± 0.2[b]
	HPH 150			49.7 ± 0.1[b]	-6.3 ± 0.1[b]	25.4 ± 0.3[b]
	HPH 150x10			46.6 ± 0.1[d]	-3.8 ± 0.1[d]	21.4 ± 0.2[b]

529 Table 2 (continues).

Sample		Appearance	Microscopy	Colour		
				L*	a*	b*
Blanched	Control			69.0 ± 3.9[a]	-15.5 ± 0.7 [a]	30.7 ± 0.9[a]
	Ground			45.1 ± 0.2[e]	-13.5 ± 0.4[c]	21.6 ± 0.8[e]
	Pre-homogenised			46.7 ± 0.1[c]	-14.3 ± 0.1[b]	28.3 ± 0.1[b]
	HPH 80			46.8 ± 0.1[c]	-14.6 ± 0.1[b]	26.9 ± 0.1[c]
	HPH 150			46.0 ± 0.1[d]	-14.4 ± 0.1[b]	26.0 ± 0.4[d]
	HPH 150x10			51.6 ± 0.4[b]	-6.7 ± 0.2[d]	22.3 ± 0.2[e]

530 [a-f] in the same column and within not-blanched and blanched samples, means indicated by different letters
531 are significantly different.

532

533 Table 3. Total bacterial count (TBC), yeast and lactic acid bacteria (LAB) load of not-blanched and
534 blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing
535 pressure and number of passes.

Sample		TBC (Log CFU/g)	Yeasts (Log CFU/g)	LAB (Log CFU/g)
Not-blanched	Ground	5.71 ± 0.31	5.48 ± 0.14	<D.L.
	Pre-homogenised	5.57 ± 0.21	5.44 ± 0.46	<D.L.
	HPH 80	4.31 ± 0.39	4.13 ± 0.55	<D.L.
	HPH 150	1.85 ± 0.22	<D.L.	<D.L.
	HPH 150x10	3.20 ± 0.10	<D.L.	<D.L.
Blanched	Ground	3.23 ± 0.54	<D.L.	<D.L.
	Pre-homogenised	3.16 ± 0.44	<D.L.	<D.L.
	HPH 80	2.00 ± 0.79	<D.L.	<D.L.
	HPH 150	3.20 ± 0.38	<D.L.	<D.L.
	HPH 150x10	2.00 ± 0.37	<D.L.	<D.L.

536 D.L. = detection limit = 1.70 Log CFU/g

Figure captions

Figure 1. Total phenolic content (TPC) (A) and polyphenoloxidase activity (Activity PPO) (B) of not-blanched and blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes.

Figure 2. Phase separation (A), and pectin methylesterase activity (Activity PME) (B) of not-blanched and blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes.

Figure 3. Viscosity of blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes.

Figure 4. Red-point (a*) of blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes, during 15-day refrigerated storage.

Figure 5. Total bacterial count (TBC) and lactic acid bacteria (LAB) load of blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes, during 15-day refrigerated storage. Detection limit = 1.7 Log CFU/g.

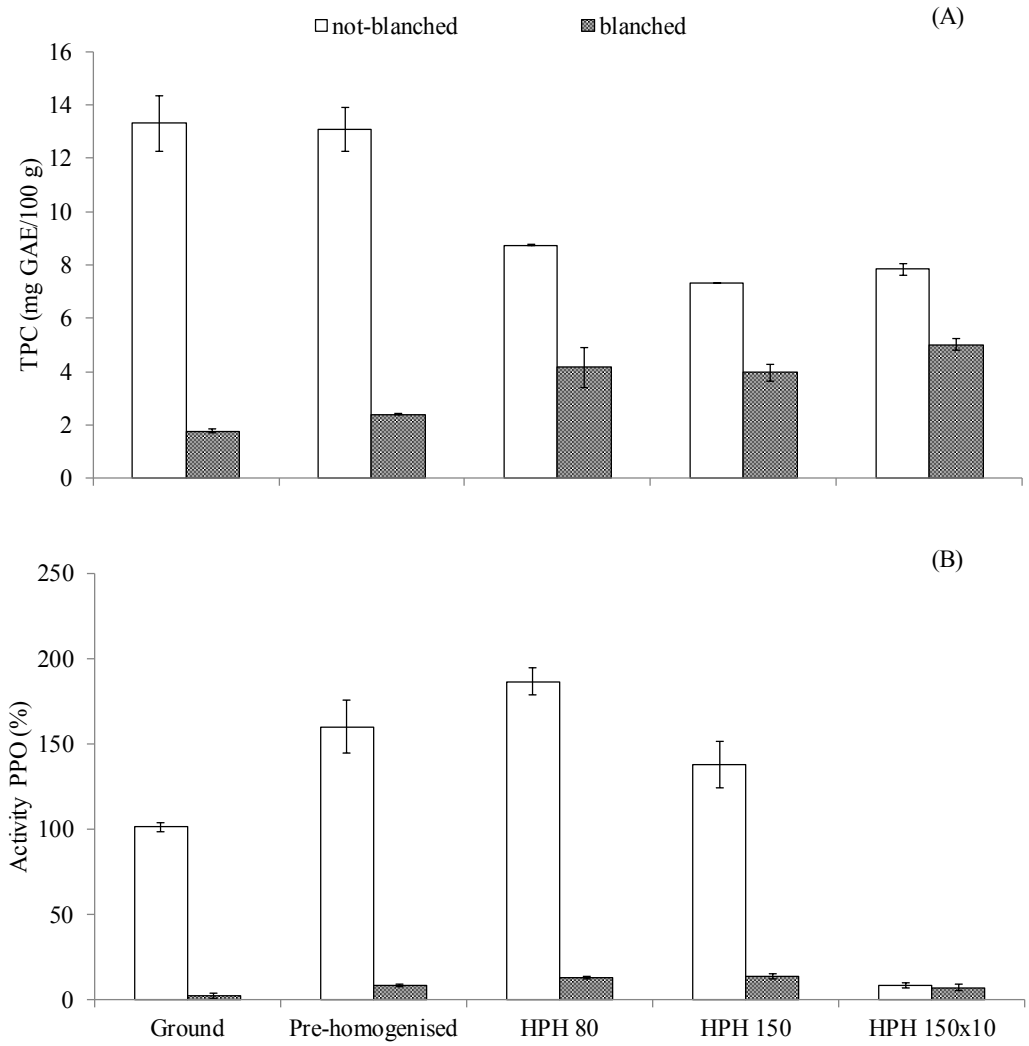


Figure 1. Total phenolic content (TPC) (A) and polyphenoloxidase activity (Activity PPO) (B) of not-blanched and blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes.

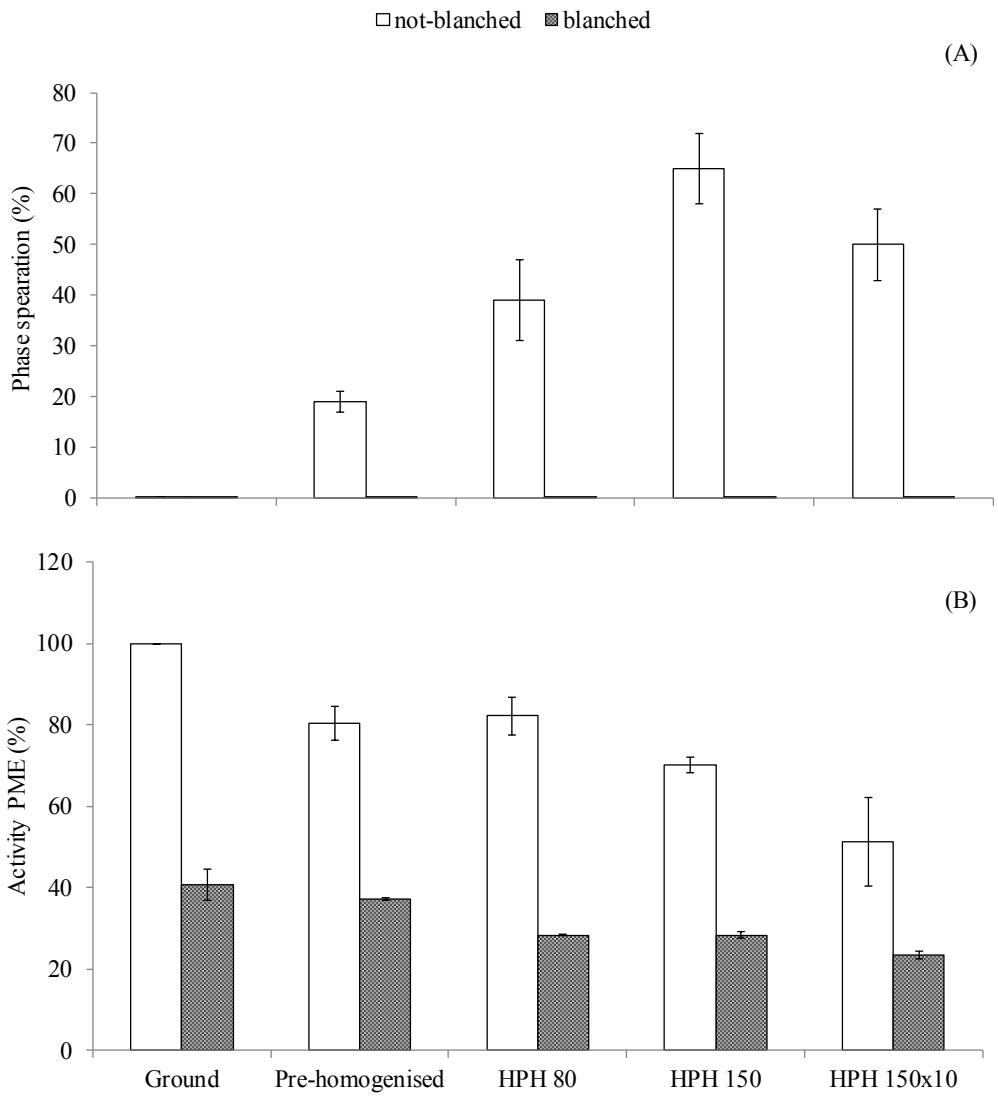


Figure 2. Phase separation (A), and pectin methylesterase activity (Activity PME) (B) of not-blanched and blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes.

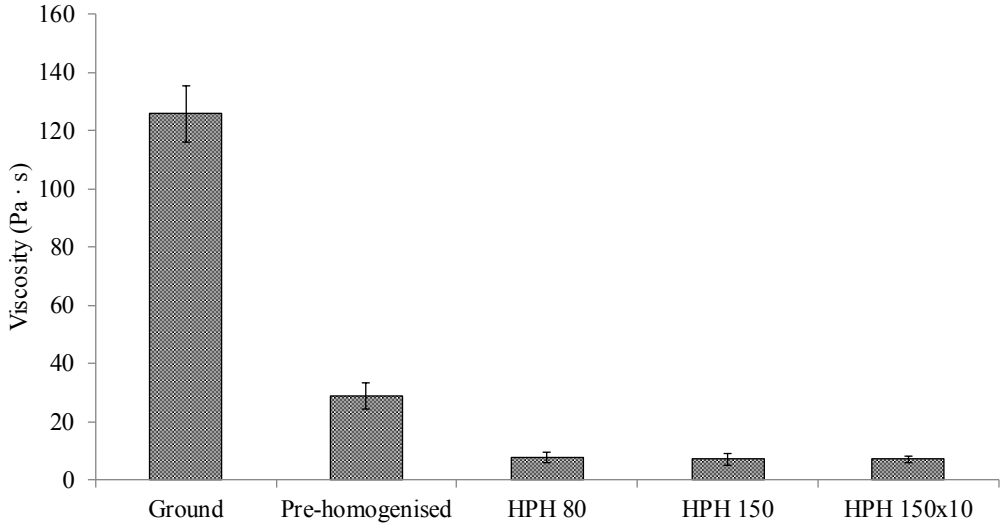


Figure 3. Viscosity of blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes.

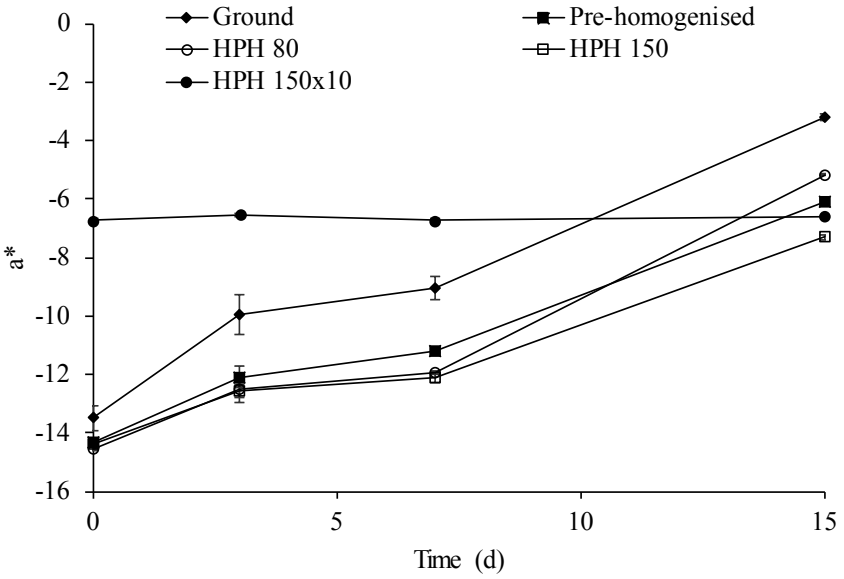


Figure 4. Red-point (a*) of blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes, during 15-day refrigerated storage.

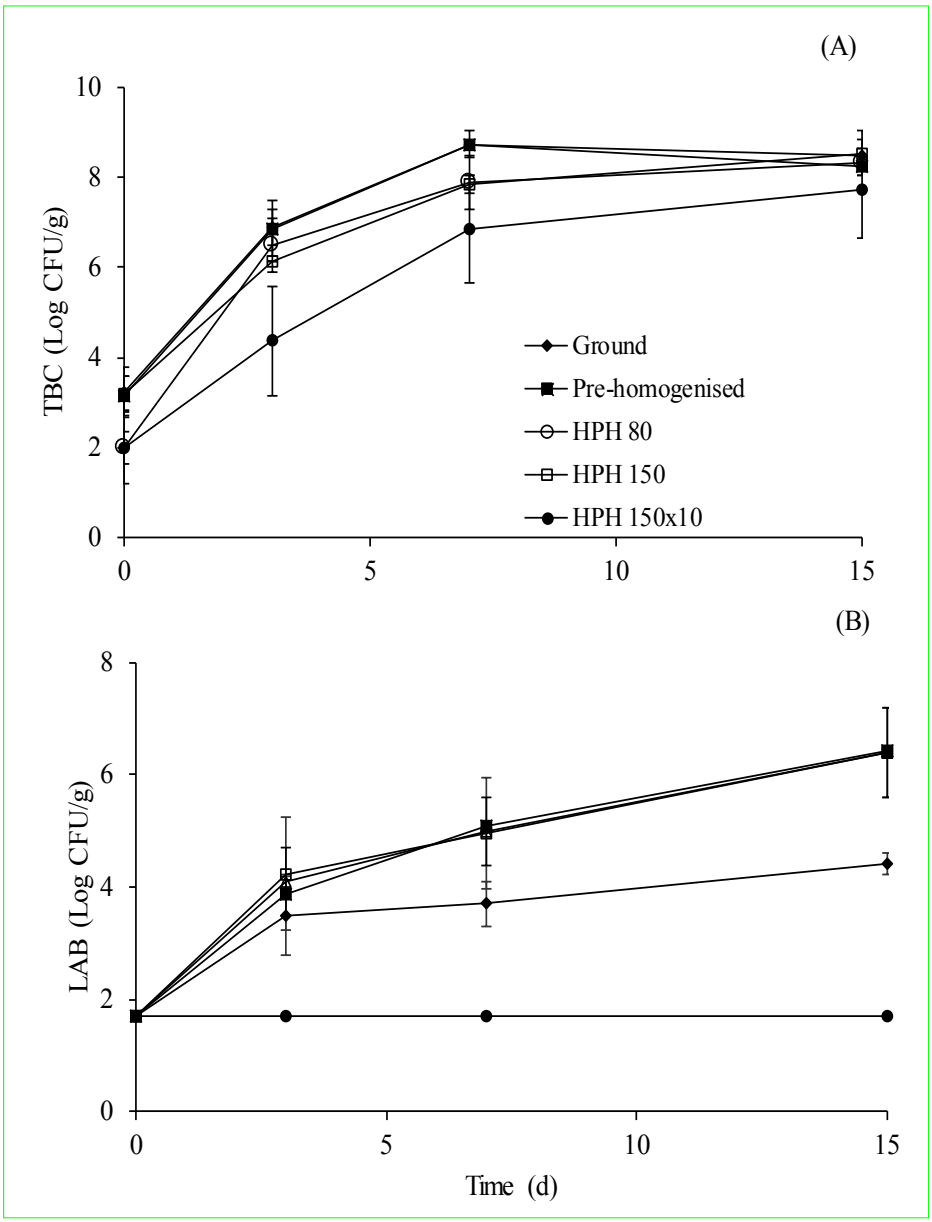


Figure 5. Total bacterial count (TBC) and lactic acid bacteria (LAB) load of blanched lettuce waste subjected to grinding, pre-homogenisation and HPH treatments at increasing pressure and number of passes, during 15-day refrigerated storage. Detection limit = 1.7 Log CFU/g.